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13. ABSTRACT *(Maximum 200 words)*

Our laboratory is studying the polyurethanase enzymes and genes that encode them from three species of *Pseudomonas*. We have purified and characterized five polyurethane hydrolyzing proteins and cloned four polyurethanase genes, in which two have been sequenced. Nucleotide sequeencing of the polyurethanase genes revealed that the deduced polypeptide sequence contains a serine-hydrolase consensus sequence GXSXG. The polypeptides lacked an N-terminal signal peptide, but did contain a short region toward the C-terminus that has been observed in secreted lipases. The most closely related proteins to the polyurethanases had amino acid sequences homologies of 70% for four lipases from *P. fluorescens* and 51% for lipases from *Serratia marcescens*. Northern blot analysis revealed that the polyurethanase genes are constitutively expressed. The results obtained from this study has yielded valuable information that is essential for an understanding of the biochemical and genetic properties of bacterial polyurethanases and how these properties may be utilized to improve polyurethane degradation. The study has a potentially major impact on understanding mechanisms by which enzymes degrade polyurethane.

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OBJECTIVE: To elucidate the enzymology and mechanism of polyurethane degradation in *Pseudomonas fluorescens, Pseudomonas chlororaphis,* and *Comamonas acidovorans.*

APPROACH: Polyurethanase proteins are purified and characterized from the various pseudomonads of interest. The purified enzymes are N-terminal sequenced and degenerate oligo-nucleotides are synthesized. The oligo-nucleotides are used to probe genomic libraries of the various pseudomonads in which to corrosponding genes can be identified. The cloned poyurethanase genes and the analyzed by DNA sequencing, RNA analysis, and expression in *E. coli*.

ACCOMPLISHMENTS:

Our laboratory is currently studying the polyurethanase enzymes and genes that encode them from three species of *Pseudomonas* (Table 1). Interestingly, the three species of bacteria produce different polyurethanase activities but are inhibited by serine hydrolase inhibitors. These data suggest that either esterase and/or protease activities are involved in the degradation of Impranil.

TABLE 1. Characteristics of Purified PUase Isolated by our Laboratory.

Bacterial Isolate	Molecular Mass	Enzyme Specificity	Inhibition	Heat Stable[1]
P. fluorescens	29 kDa	Protease	PMSF	+
P. fluorescens	48 kDa[2,3]	Esterase	PMSF	-
P. chlororaphis	63 kDa[2,3]	Esterase/Protease	PMSF	+
P. chlororaphis	31 kDa	Esterase	PMSF	+
P. chlororaphis	70 kDa[2]	Esterase	PMSF	-
C. acidovorans	42 kDa	Esterase/Protease	PMSF/TI	+

[1]Enzyme activity (100%) after 10 min at 100°C

[2] Enzyme has been cloned and expressed in *E. coli*

[3] Gene has been sequenced

Growth of *Comamonas acidovorans* on polyurethane resulted in obtaining growth parameters of K_S and μ_{max} values of 0.3 mg·ml^{-1} and 0.7 doublings·h^{-1} respectfully (Allen *et al.*, 1999). Our laboratory has purified and characterized a 42kDa PUase enzyme displaying esterase/protease activity (Allen *et al.*, 1999). Nakajima-Kambe *et al.* (1995, 1997) reported a strain of *Comamonas acidovorans* that could utilize solid polyester PU as the sole carbon and nitrogen source. These authors indicated the role of an extracellular membrane bound esterase

activity in PU degradation. Purification of the membrane bound esterase revealed a thermally labile protein having a 62 kDa molecular mass (Akutsu *et al.*, 1998).

Growth of *Pseudomonas chlororaphis* on polyurethane resulted in obtaining growth parameters of K_S and μ_{max} values of 0.9 mg·ml^{-1} and 1.3 doublings·h^{-1} respectfully (Ruiz *et al.*, 1999). Two PUase enzymes have been purified and characterized, a 65 kDa esterase/protease and a 31 kDa esterase (Ruiz *et al.*, 1999). In addition to the enzymology, a PUase gene encoding a 63 kDa protein has been cloned in *E. coli*. The gene encoding the 63 kDa PUase, *pueA*, has been expressed and sequenced (Genebank, Accession AF069748). An open reading frame (ORF), 1,854 nucleotides in length, was observed in the cloned insert by codon analysis, starting with an AUG codon in nucleotide 175 and stopping with an UGA codon in nucleotide 2,029. Identity searching in database with the BLAST program indicated a strong similarity of this ORF to several lipase genes. Therefore, the sequenced gene was called *pueA* (polyurethanase-esterase A). The initiating AUG codon was preceded 7 nucleotides by a plausible ribosomal binding site with an AAGAGG. The deduced protein PueA has 617 amino acid residues and a molecular mass of 65 kDa. The encoded amino acid sequence of PueA was aligned with six other extracelluar lipases from *Pseudomonas fluorescens* and *Serratia marcescens*. The PueA amino acid sequence showed a high identity with these lipases (ranging from 58 to 75%). Two highly conserved regions were observed for PueA and the extracelluar lipases (Figure 1). A putative catalytic domain for serine hydrolases, amino acids 203-211, was observed. The sequence motif, G-H-S-L-G, as well as the location were conserved among all seven polypeptides. In addition, a second conserved region was observed for PueA and the six lipases. A common motif among secreted lipases was observed at amino acids 364 to 400. The secretion sequence motif, G-G-X-G-X-D-X-X-E was conserved as well as the location. Interestingly, the location of the secretion motif is consistently 163 amino acid residues downstream from the putative catalytic motif for each of the seven polypeptides. The size of the *pueA* mRNA was determined by Northern blot analysis. A 1900 nucleotide transcript was detected from *P. chlororaphis* grown in various carbon sources. The transcript analysis indicates that the *pueA* gene is constitutively expressed. The gene encoding the 31kDa protein has not been isolated, however the *P. chlororaphis* library generated is currently being screened for its presence.

Growth of *Pseudomonas fluorescens* on polyurethane resulted in obtaining growth parameters of K_S and μ_{max} values of 0.9mg·ml^{-1} and 1.6 doublings·h^{-1} respectfully (Howard and Blake, 1999). Two PUase enzymes have been purified and characterized from this bacterial isolate, a 29kDa protease (Howard and Blake, 1999) and a 48kDa esterase (Vega *et al.*, 1999). In addition, to the enzymology of the PUases the gene encoding a 48 kDa protein has been cloned and expressed in *E. coli* (Vega *et al.*, 1999). The gene encoding PulA has been sequenced (GeneBank AF144089). The deduced amino acid sequence has 461 amino acid residues and a molecular mass of 49 kDa. The PulA amino acid sequence showed high identity with the six lipases (58 to 75%) and PueA from *P. chlororaphis* (85%). The size of the *pulA* mRNA was determined by Northern blot analysis. A 1500 nucleotide transcript was detected from *P. fluorescens* grown in various carbon sources. The transcript analysis indicates that the *pulA* gene is constitutively expressed. The gene encoding for the 29kDa protease has not been isolated, however the *P. fluorescens* library generated is currently being screened for its presence.

Figure 1. Comparison of putative active sites and secretion sites for the PueA from *P. chlororaphis* and six extracellular lipases. The numbers indicate the position of the amino acid residues within the protein sequence. Percent identity of complete gene when compared to PueA is indicated. Panel A, Active site region with identical residues boxed. Panel B, Secretion signal region with identical residues shaded.

A

		Active Site										% Identity
Pseudomonas fluorescens SIK W1	202-	Val	Ser	Gly	His	Ser	Leu	Gly	Gly	Leu	-210	58
Pseudomonas fluorescens B52	203-	Val	Ser	Gly	His	Ser	Leu	Gly	Gly	Leu	-211	70
Pseudomonas LS107d2	203-	Val	Ser	Gly	His	Ser	Leu	Gly	Gly	Leu	-211	67
Serratia marcescens SM6	203-	Val	Ser	Gly	His	Ser	Leu	Gly	Gly	Leu	-211	61
Serratia marcescens Sr41	202-	Ile	Ser	Gly	His	Ser	Leu	Gly	Gly	Leu	-210	61
Pseudomonas chlororaphis	203-	Val	Ser	Gly	His	Ser	Leu	Gly	Gly	Leu	-211	- -
Pseudomonas fluorescens	180-	Val	Ser	Gly	His	Ser	Leu	Gly	Gly	Met	-188	85

B

Serratia marcescens SM6	364-	E	T	H	S	G	P	T	F	I	I	G	S	D	G	N	D	L	I
Serratia marcescens Sr41	363-	E	T	H	S	G	P	T	F	I	I	G	S	D	G	N	D	L	I
Pseudomonas fluorescens SIK W1	363-	E	P	H	T	G	N	T	F	I	I	G	S	D	G	N	D	L	I
Pseudomonas fluorescens B52	363-	E	P	H	K	G	N	T	F	I	I	G	S	D	G	N	D	L	I
Pseudomonas LS107d2	363-	E	P	H	K	G	D	T	F	I	I	G	S	A	G	N	D	L	I
Pseudomonas chlororaphis	364-	E	T	H	K	G	S	T	F	I	I	G	S	D	G	N	D	L	I
Pseudomonas fluorescens	340-	E	A	H	K	G	N	T	F	I	I	G	S	D	G	D	D	L	I

Serratia marcescens SM6	364-	K	G	G	K	G	N	D	Y	L	E	G	R	D	G	D	D	I	F	R	-400
Serratia marcescens Sr41	363-	K	G	G	K	G	N	D	Y	L	E	G	R	D	G	D	D	I	F	R	-399
Pseudomonas fluorescens SIK W1	363-	Q	G	G	K	G	A	D	F	I	E	G	G	K	G	N	D	T	I	R	-399
Pseudomonas fluorescens B52	363-	Q	G	G	N	G	A	D	F	I	E	G	G	K	G	N	D	T	I	R	-399
Pseudomonas LS107d2	363-	Q	G	G	K	G	A	D	F	I	E	A	A	K	G	N	D	T	I	R	-399
Pseudomonas chlororaphis	364-	K	G	S	T	F	I	I	G	S	D	G	N	D	L	I	Q	G	G	S	-400
Pseudomonas fluorescens	340-	K	G	N	T	F	I	I	G	S	D	G	D	D	L	I	K	G	G	R	-376

Consensus Sequence G GX GX D X X X

SIGNIFICANCE:

The goal of the proposed research is to dissect the polyurethanase enzymes using an integrated enzymologic and genetic approach. The proposed study will involve appropriate and innovative strategies for studying each of the single enzymes and genes involved in the degradation of polyurethane. The genes and gene products responsible for the degradation of polyurethane can then be identified and characterized biochemically and genetically. Thus, the proposed studies should culminate in development of a model system in which the role of single genes and gene products in polyurethane degradation can be assessed. The results obtained from this study will yield valuable information that is essential for an understanding of the biochemical and genetic properties of bacterial polyurethanase and how these properties may be utilized to improve polyurethane degradation. More specifically, characterization of the enzymology of polyurethanases will be instrumental to understanding the mechanism involved in polyurethane degradation by bacteria. A more efficient degradation of polyurethane waste biomass will provide a major contribution to both the environment and the consumer.

The long term goals of this research is to initiate a program aimed at a better understanding of the mechanism of polyurethane degradation by bacteria. One approach is to study each enzyme and gene at the single gene level using an integrated enzymological, biochemical, physical, and genetic program. **The proposed studies should culminate in development of a novel anaerobic model system in which the role of single genes and gene products in polyurethane degradation can be assessed, and the degradation products established .**

PUBLICATIONS AND ABSTRACTS:

Howard, G.T. and R.C. Blake. 1999. Growth of *Pseudomonas fluorescens* on a polyester-polyurethane and the purification and characterization of a polyurethanase-protease enzyme. International Biodeterioration and Biodegradation. **42:** 213-220.

Ruiz, C., N. Hilliard, and **G.T. Howard**. 1999. Growth of *Pseudomonas chlororaphis* on a polyester-polyurethane and the purification and characterization of a polyurethanse-esterase enzyme. International Biodeterioration and Biodegradation. **43:** 7-12.

Howard, G.T. and N.P. Hilliard. 1999. Use of coomassie blue-polyurethane interaction in detection of polyurethanease proteins and polyurethanolytic bacteria. Int. Bioterioration Biodegradation **43:**23-30

Allen, A., N. Hilliard, and **G.T. Howard**. 1999. Purification and characterization of a soluble polyurethane degrading enzyme from *Comamonos acidovorans*. International Biodeterioration and Biodegradation. **43:** 37-41.

Ruiz, C.,T. Main, N. Hilliard, and **G.T. Howard**. 1999. Purification and characterization of two polyurethanse enzymes from *Pseudomonas chlororaphis*. . International Biodeterioration and Biodegradation. **43:** 43-47.

Vega, R., T. Main, and **G.T. Howard**. 1999. Cloning and expression in *Escherichia coli* of a polyurethane-degrading enzyme from *Pseudomonas fluorescens*. International Biodeterioration and Biodegradation. **43:** 49-55.

Ruiz, C. and **G.T. Howard**. 1999. Nucleotide sequencing and transcriptional regulation of a polyurethanase gene (*pulA*) from enzyme from *Pseudomonas fluorescens*. International Biodeterioration and Biodegradation. Submitted for publication.

Stern, R.V. and **G.T. Howard**. 1999. Cloning,Over-expression and Nucleotide Sequence of a Gene (*pueA*) Encoding a Polyurethanase from *Pseudomonas chlororaphis*. FEMS Microbiolgy Letters. Submitted for publication.

Ruiz, C. and **G. T. Howard**. 1997 Characterization of Growth and a Polyurethanase protein from *Pseudomonas chlororaphis*. Abstr. Ann. Meet. Amer. Soc. Microbiol.

Main, T., N. Hilliard, **G. Howard**. 1998. Purification and Characterization of a polyurethanse from *Pseudomonas chlororaphis*. Abstr. Ann. Meet. Amer. Soc. Microbiol.

Allen, A.B., N.P. Hilliard, and **G.T. Howard**. 1998. Purification and characterization of a polyurethane degrading enzyme from *Comamonas acidovorans*. Abstr. Ann. Meet. Amer. Soc. Microbiol.

Stern, R.V. and **G.T. Howard**. 1998. Cloning and expression of a polyurethane gene from *Pseudomonas chlororaphis*. Abstr. Ann. Meet. Amer. Soc. Microbiol.

Ruiz, C.R., N.P. Hilliard, and **G.T, Howard**. 1998. Purification and characterization of a polyurethanse from *Pseudomonas chlororaphis*. Abstr. Ann. Meet. Amer. Soc. Microbiol.

Vega, R.E. and **G.T. Howard**. 1998. Cloning and expression of a polyurethanse gene from *Pseudomonas fluorescens*. Abstr. Ann. Meet. Amer. Soc. Microbiol.

Vega, R.E. and **G.T. Howard**. 1998. Cloning and expression of a polyurethanse gene from *Pseudomonas fluorescens*. Ann. Meet. Amer. Soc. Microbiol. South Central Branch

Ruiz, C. and **G.T. Howard**. 1999. Nucleotide sequencing and transcriptional analysis of a polyurethanase gene from *Pseudomonas fluorescens* . Abstr. Ann. Meet. Amer. Soc. Microbiol.

Stern, R. and **G.T. Howard**. 1999. Nucleotide sequence analysis and overexpression of the polyurethanase encoded by the gene (pueA) from *Pseudomonas chlororaphis*. Abstr. Ann. Meet. Amer. Soc. Microbiol.

Main, T. and **G.T. Howard**. 1999. Purification and characterization of a polyurethanase from *Pseudomonas fluoirescens*. Abstr. Ann. Meet. Amer. Soc. Microbiol.

Vicknair, J.S. and **G.T. Howard**. 1999. Cloning and expression of a polyurethanase gene from *Pseudomonas chlororaphis*. Abstr. Ann. Meet. Amer. Soc. Microbiol.